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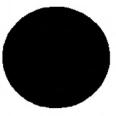
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Patents Form 1/77

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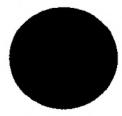
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Protein Production Process

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The present invention relates to a process for the preparation of a therapeutic protein complex, and to apparatus and reagents for use in the process.

10

A multimeric form of α -lactalbumin or MAL has previously been reported as having therapeutic applications both in the field of antibiotic (WO96/04929) and cancer therapy (A. Håkansson et al., Proc. Natl. Acad. Sci USA, (1995) 92, 8064-8068).

15

MAL was obtained by passing a casein fraction of milk, particularly human milk, down an ion exchange column, specifically a DEAE-Tris-acyl M column using an NaCl gradient. A pool containing MAL which was active therapeutically was obtained. In addition, the conversion of commercially available monomeric α -lactalbumin to the multimeric form was reported.

20
25 The applicants have found an improved way of producing MAL in greater yields and from a greater variety of α -lactalbumin sources.

30 The present invention provides a method for producing a multimeric form of α -lactalbumin which comprises exposing a source of α -lactalbumin to an ion exchange medium which has been pre-treated with casein or an active component thereof and recovering α -lactalbumin in a multimeric form therefrom.

35 Pre-treatment or "training" the ion exchange medium with casein or active components thereof has been found to be particularly effective in increasing the yield of MAL, for

example from various sources such as commercially available α -lactalbumin derived from both human and bovine milk.

The expression "active component" used herein refers to those
5 one or more elements found in casein which produce the desired
improvement in the process when used as a pre-treatment of the
ion exchange column. It is known for instance, that casein
contains a number of lipids and occasionally free fatty acids.
The fatty acid content of casein is marked increased if for
10 example, the casein is frozen or subjected to hydrolysis
reactions. It is possible that these components are retained
on the column and give rise to the improved MAL production
capability observed.

15 Casein from human milk contains polyunsaturated fatty acids
like oleic acid, linoleic, γ -linoleic and arachidonic acid as
well as triglycerides and lipids particularly phospholipids
such as sphingomyelin. Any of these either alone or in
combination, may produce similar enhancement effects on the
20 column.

It would be a matter of routine to test which of these
components or combination of components produced enhanced
effects and then the reagents could be used alone in place of
25 the casein in order to pre-treat the ion exchange material.

The casein or active component used in the pre-treatment step
may be isolated from milk derived from various mammals, such
as humans, bovines, sheep or goats. Preferably however the
30 casein or active component used in the pre-treatment are
derived from human milk.

Isolation of casein fractions can be carried out using known
methods, for example as described in WO 96/04929. The casein
35 may be used directly or it may be frozen and later thawed
prior to use. It has been found that casein which has been
frozen or derived from frozen human milk is preferred in the
pre-treatment step. Where the casein is used directly, it is

preferable that it is first subjected to hydrolysis so as to hydrolyse some triglycerides present and so increase the amount of free fatty acid present before use in the pre-treatment step. This hydrolysis may be achieved for example by exposure of the casein to bile salts (J. Bitman et al., J. Ped. Gast. Nutr. 1983: 521-524).

Preferably the ion-exchange material used is arranged in a column as is conventional in the art. The various treatments can then be eluted through the column.

Suitably casein or the active components thereof are eluted through a column containing new unused ion exchange material such as DEAE Trisacryl. Suitable elution buffers include Tris-HCl with a pH of 8.5.

The amount of casein or active components applied to the column in this way may be small depending upon the volume of α -lactalbumin is required to be converted to MAL. For example, it has been found that only 30mg of casein or casein equivalents per ml of column material can be used in the conversion of multiple 10mg runs of bovine α -lactalbumin. After 6 runs (60mg), the yield began to decrease, but some multimeric α -lactalbumin was still obtained even after 10 runs.

In a preferred embodiment, the column is then washed with ion exchange buffer, such as the Tris-HCl buffer mentioned above, without casein, and preferably also other buffers which are to be used in the process, such as a NaCl containing buffer to ensure that nothing unspecific will elute from the column when used in the process. Washing may be done several times.

The column may then be eluted with the source of α -lactalbumin as described above dissolved in the ion exchange buffer. The column comprises anion exchange material. Suitably a salt concentration gradient is induced in the column by elution

with buffer containing suitable salts, for example those containing a suitable anion such as chloride. One such salt is sodium chloride. MAL containing fractions can then be isolated from the column. These fractions may be identified 5 spectroscopically for example as illustrated hereinafter.

In the experiments illustrated hereinafter, it has been found that in general, the most active MAL fraction elutes first although some may be carried over into a second elution peak.

10 Using the process of the invention, MAL can be obtained from a wide variety of α -lactalbumin sources. For example it may be isolated from casein fractions or whey fractions of milk from any of the above-mentioned mammals or from commercially 15 available monomeric α -lactalbumin which has been derived from any of these mammals. For example, monomeric bovine α -lactalbumin can be converted to therapeutic MAL in good yields, in some cases substantially completely, by treatment in accordance with the invention.

20 In some instances, depending upon the purification process used in its production, it may be preferable to contact the α -lactalbumin with a calcium chelating agent such as EDTA (ethylene diamine tetraacetic acid) in order to remove excess 25 calcium. This may be applied as a pre-treatment in which the α -lactalbumin is contacted with the chelating agent prior to elution down the ion exchange column, or alternatively, the EDTA may be added to the elution buffer. Whether or not this is necessary in order to obtain optimum yields of active MAL 30 can be determined by carrying out trial runs as illustrated hereinafter.

A pre-treated column can be used repeatedly to convert numerous fractions of α -lactalbumin to MAL. Once the column 35 is exhausted or the conversion rate drops to unacceptable levels, the pre-treatment step can be repeated in order to restore the enhanced MAL production activity.

The reason why the "training" of the column in this way is so advantageous in MAL production is not entirely clear. It is possible that one or more components of the casein are retained on the column and thereafter catalyse MAL production or stabilise α -lactalbumin in its multimeric form.

Ion-exchange media and columns which have been trained or conditioned in this way form a further aspect of the invention, as does multimeric α -lactalbumin obtainable using this method.

The invention will now be particularly described by way of example with reference to the accompanying diagrammatic drawings in which:

Figure 1 shows an ion exchange chromatogram obtained by elution of a sample of pre-treated human monomeric α -lactalbumin down a clean ion exchange column:

Figure 2 shows an ion exchange chromatogram obtained by elution of a sample of pre-treated-human monomeric α -lactalbumin and lipids down a clean ion exchange column:

Figure 3 shows an ion exchange chromatogram obtained by elution of a sample of human monomeric α -lactalbumin down a trained ion exchange column in accordance with the invention: and

Figure 4 shows an ion exchange chromatogram obtained by elution of a sample of bovine monomeric α -lactalbumin down a trained ion exchange column in accordance with the invention.

In the Figures, the protein peak is indicated by a thick line. The ion exchange matrix used in the following examples was DEAE Trisacryl M from BioSepa, France. The buffers

comprised: Buffer A, 10mM Tris-HCl pH 8.5 and Buffer B, 10 mM Tris-HCl with 1M NaCl pH 8.5.

The experiments were carried out at room temperature (22°).

5 Sample was dissolved in 10ml of buffer A.

Sample solution were injected onto the column at 1 ml/minute. The column was then eluted with buffer A for 10 minutes in order to get the sample on the column. Then a gradient of 10 buffer B from 15-30% was induced. The gradient was held at 30% buffer B for 20 minutes during which time unwanted protein was eluted from the column. The concentration of buffer B was then increased to 100% which was held for 10 minutes. Where present, a MAL containing fraction eluted from the 15 column during this period. The concentration of buffer B was reduced to 0% which was held for 20 minutes. Thereafter the concentration of buffer B was raised again to 100% and held for 20 minutes, during which a second MAL containing fraction was eluted. The concentration of buffer B was then 20 reduced to 0% and the column eluted with buffer A only for a further 50 minutes.

Product was analysed using gel electrophoresis (Tris-Glycine PAGE gels 4-20%) as described previously in WO 96/04929, the 25 content of which is hereby incorporated by reference.

Comparative Example A

This example was carried out using a new and previously unused ion exchange matrix. Monomeric α -lactalbumin (ca. 25mg) was 30 added to the column in the manner outlined above. No multimeric peaks were obtained on this occasion.

Monomeric α -lactalbumin (20mg) was subjected to a procedure used in the precipitation of casein. Specifically, the 35 sample was mixed with 10% potassium oxalate and incubated overnight at 4°C. The pH was then lowered to 4.3 and the sample incubated at 32°C for 2 hours. After o.n. incubation at

4°C, the sample was added to the column. Although small multimeric peaks were detected (Figure 1), no protein was found after dialysis and freeze drying of the product.

5 Monomeric α -lactalbumin (20mg) was mixed with lipids extracted from whole human milk, subjected to the casein precipitation procedure outlined above, and added to the column. Again small multimeric peaks were detected but no protein was found after dialysis and freeze drying (Figure 2).

10

Example 1

In this example, 300mg of casein derived from human milk was run on a fresh unused ion exchange matrix. The matrix was then washed with two runs of buffer A. Untreated monomeric human α -lactalbumin (8mg) was added to the column. Two multimeric peaks were found (Figure 3). Four further samples were run down this column and all gave two multimeric peaks.

Comparative Example B

20 Example A above was repeated using a different sample of unused matrix and with monomeric bovine α -lactalbumin samples. None of the samples gave any multimeric peaks.

Example 2

25 Example 1 above was repeated using monomeric bovine α -lactalbumin in place of human α -lactalbumin. Two clear multimeric peaks were obtained (Figure 4).

Example 3

30 Supplies of α -lactalbumin were obtained from various sources including commercially available human and bovine α -lactalbumin (Sigma) and α -lactalbumin isolated from human milk by ammonium sulphate precipitation of all proteins except α -lactalbumin, and then running the supernatant on a phenyl-sepharose column.

The samples were eluted down a column as described in Example 1. A second group of samples were pre-treated with EDTA by adding 7mg of the α -lactalbumin to 10ml of Buffer A containing 1mM of EDTA. The mixture was left at room temperature for about 3 hours and was then added to the trained column and run using the buffers set out in Example 1.

A third group of samples were treated with an EDTA containing buffer as described above and then run using EDTA buffers.

The two peaks which are believed to contain MAL were kept separately and tested individually. Representative results are set out in Table 1.

Table 1

<u>Source of α-lactalbumin</u>	<u>EDTA addition</u>	<u>MAL Peak tested</u>	<u>Dosage</u>	<u>Viability of tumour cells*</u>
Commercial	Untreated	1	0.5mg/ml	0%
			1.0mg/ml	0%
human α -lactalbumin		2	1.0mg/ml	0%
Human α -lactalbumin +	Untreated	1	1.0mg/ml	79%
		2	1.0mg/ml	95%
	EDTA pre-treated	1	1.0mg/ml	0%
		2	ca.1.5mg /ml	98%
	EDTA pre-treatment +	1	0.5mg/ml	0%
			1.0mg/ml	0%
	EDTA buffer	2	0.5mg/ml	89%
			1.0mg/ml	0%
Commercial	untreated	1	0.3mg/ml	50%
			0.5mg/ml	0%
bovine α -lactalbumin		2	0.5mg/ml	98%
			1.0mg/ml	98%

* Cell viability of tumour cells incubated with MAL as described below.

+ Obtained using ammonium sulphate precipitation step as discussed above.

These results indicate, that the first MAL containing fraction to elute is the most active. It is possible, that the second peak contains only some residual MAL which 'leaked' from the first peak.

With certain samples, and in particular, those isolated from human milk using an ammonium sulphate precipitation step as outlined above, the removal of some calcium is necessary in order to ensure efficient conversion of monomeric α -lactalbumin to MAL.

Biological Data

The biological efficacy of the products obtained in the above Examples was tested using tumour cell lines as described in . Cell lines were incubated with samples of the products at various concentrations in cell culture media at 37°C for various time points. After incubation, the cells are harvested and the cell viability determined using a vital dye (trypan blue). DNA was then prepared from the cells which was subjected to agarose gel electrophoresis. Analysis of the results revealed the occurrence of DNA fragmentation in all cases, indicative of apoptosis.

The activity of the products of the above Examples 1 and 2 was similar to that obtained using protein isolated from human milk casein as described by Håkansson et al (1995) supra.

Claims

1. A method for producing a multimeric form of α -lactalbumin which comprises exposing a source of α -lactalbumin to an ion exchange medium which has been pre-treated with casein or an active component thereof and recovering α -lactalbumin in a multimeric form therefrom.
2. A method according to claim 1 wherein the ion exchange medium has been treated with casein derived from human milk.
3. A method according to claim 1 or claim 2 wherein the ion exchange medium has been treated with casein which has been previously frozen or is derived from frozen milk.
4. A method according to claim 1 or claim 2 wherein the casein used in the pre-treatment of the ion exchange medium has been subjected to hydrolysis.
5. A method according to any one of the preceding claims wherein the ion exchange medium is arranged in a column.
6. A method according to any one of the preceding claims wherein the ion exchange medium comprise DEAE Trisacryl.
7. A method according to any one of the preceding claims which comprises eluting an ion exchange column with casein or the active components thereof in an ion exchange buffer, washing the column with ion exchange buffer, and then eluting the column with a source of α -lactalbumin dissolved in the ion exchange buffer in the presence of a salt concentration gradient.
8. A method according to claim 7 wherein the ion exchange buffer is Tris-HCl.

9. A method according to claim 7 or claim 8 wherein the salt concentration gradient is produced using an ion exchange buffer in which sodium chloride is dissolved.

5 10. A method according to claim 8 wherein the column is washed by elution of ion exchange buffer twice.

11. A method according to any one of the preceding claims wherein the said source of α -lactalbumin comprises monomeric
10 bovine α -lactalbumin.

12. A method according to any one of the preceding claims wherein the said source of α -lactalbumin comprises monomeric human α -lactalbumin.

15 13. An ion exchange medium for use in the method of any one of the preceding claims, said medium having been treated with casein or an active component thereof.

20 14. An ion exchange column which comprises ion exchange medium according to claim 13.

15. A multimeric form of α -lactalbumin obtained by a method according to any one of claims 1 to 12.

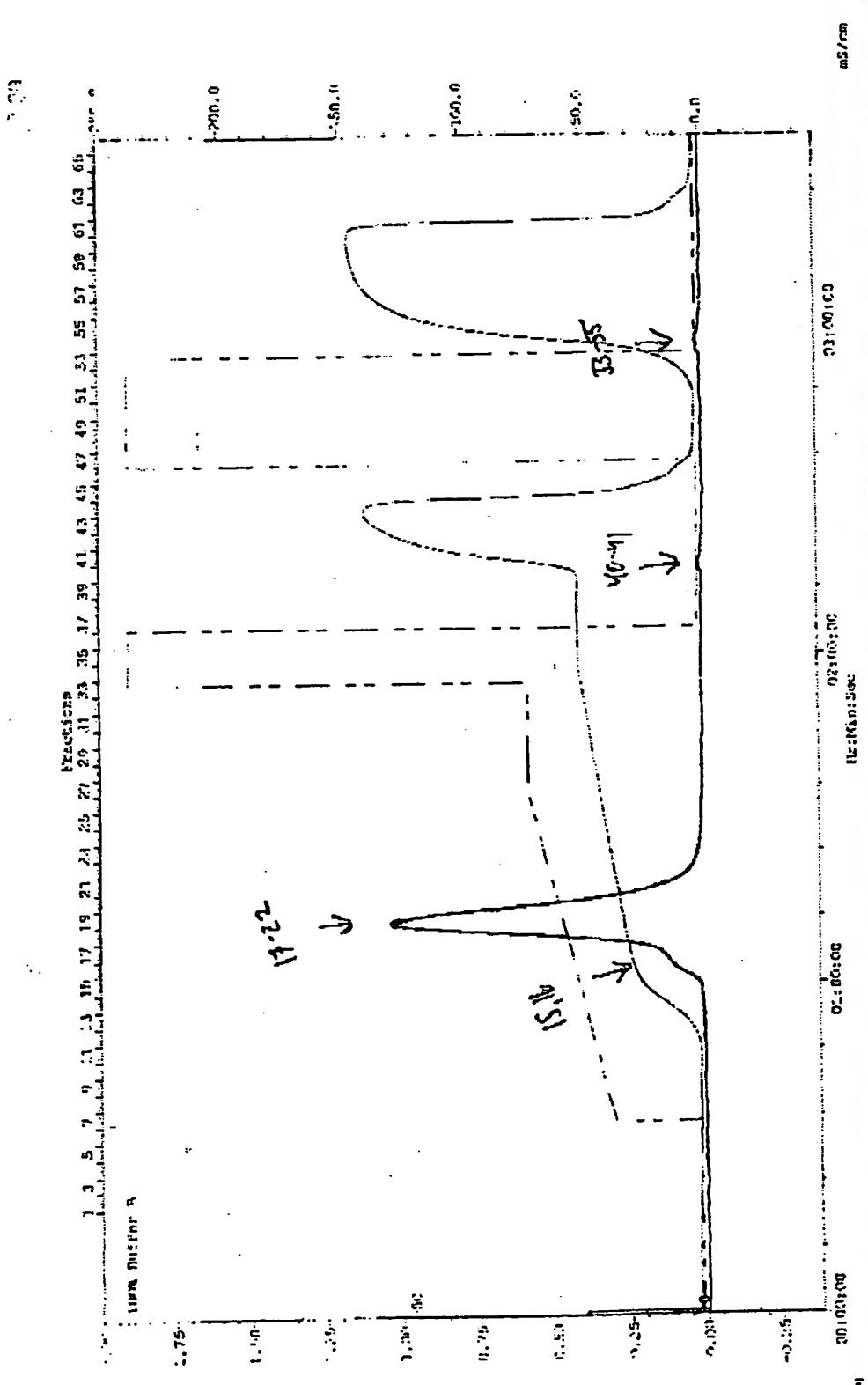
Abstract

A method for producing a multimeric form of α -lactalbumin which comprises exposing a source of α -lactalbumin to an ion exchange medium which has been pre-treated with casein or an active component thereof, and recovering α -lactalbumin in a multimeric form therefrom. Pre-treatment of the ion exchange medium, particularly with casein derived from human milk, has been found to significantly improve yields of the multimeric 10 form of α -lactalbumin and mean that it can readily isolated from readily available sources such as bovine α -lactalbumin.

This form of α -lactalbumin is useful therapeutically, in particular as an antibacterial agent and also as an anti-15 cancer therapeutic.

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Figure 1



Sample: 10 ml. Loaded through pump A

Column: Pharmacia DEAE, 1.6x20 cm.

Buffer A: Tris 10 mM, pH 8.5

Buffer B: Tris 10 mM + 1M NaCl, pH 8.5

Flow Rate: 1 ml/min

Gradient:

Chart Speed:

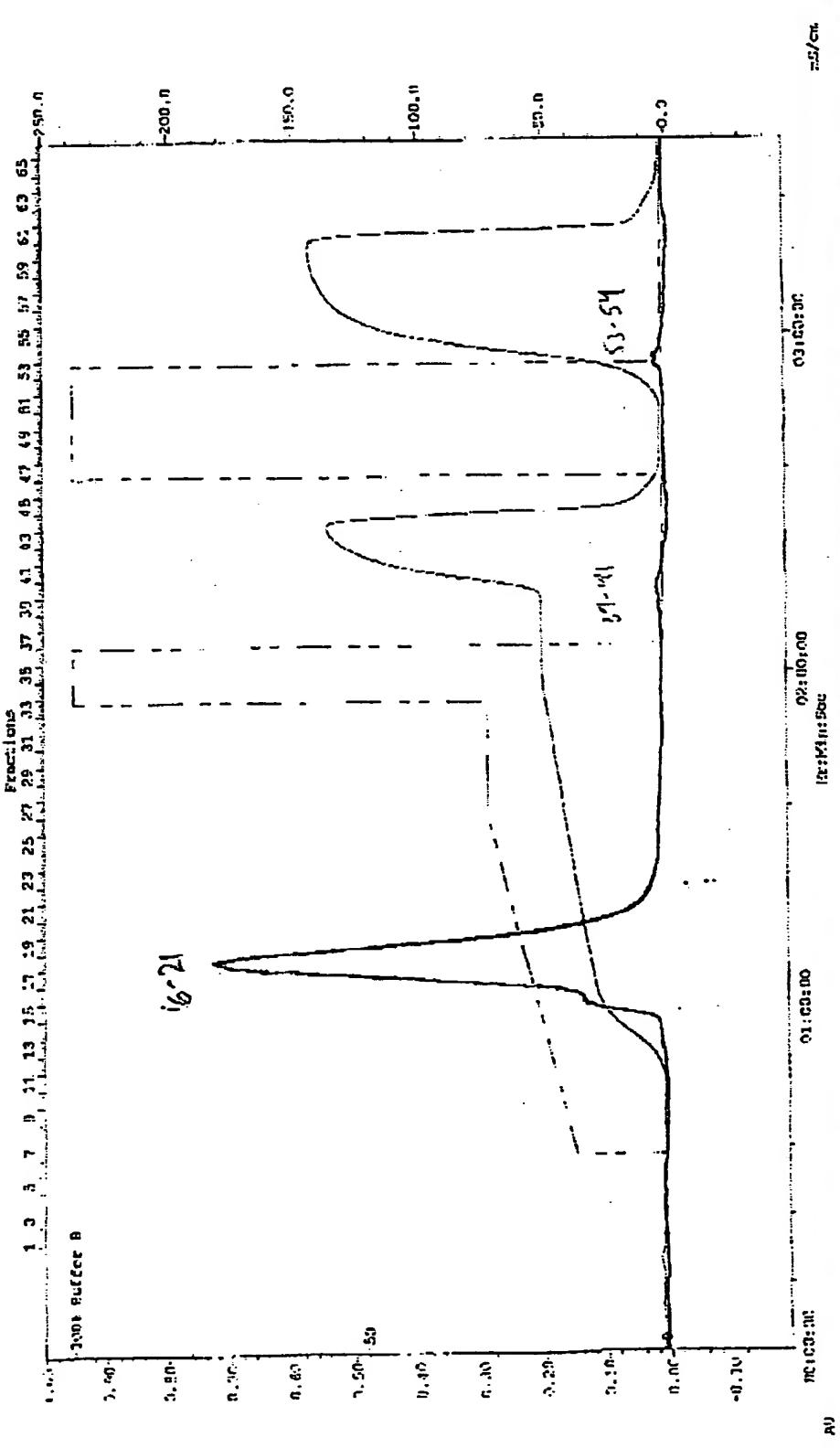
Fraction Size: 3 ml

Run Description: HaAL failt enligt casen i protokoll



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FIGURE 2



Sample: 10 ml. Loaded through pump A

Column: Pharmacia DEAE, 1.8x20 cm.

Buffer A: Tris 10 mM, pH 8.5

Buffer B: Tris 10mM + 1MNaCl, pH 8.5

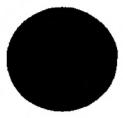
Flow Rate: 1 ml/min

Gradient:

Chart Speed:

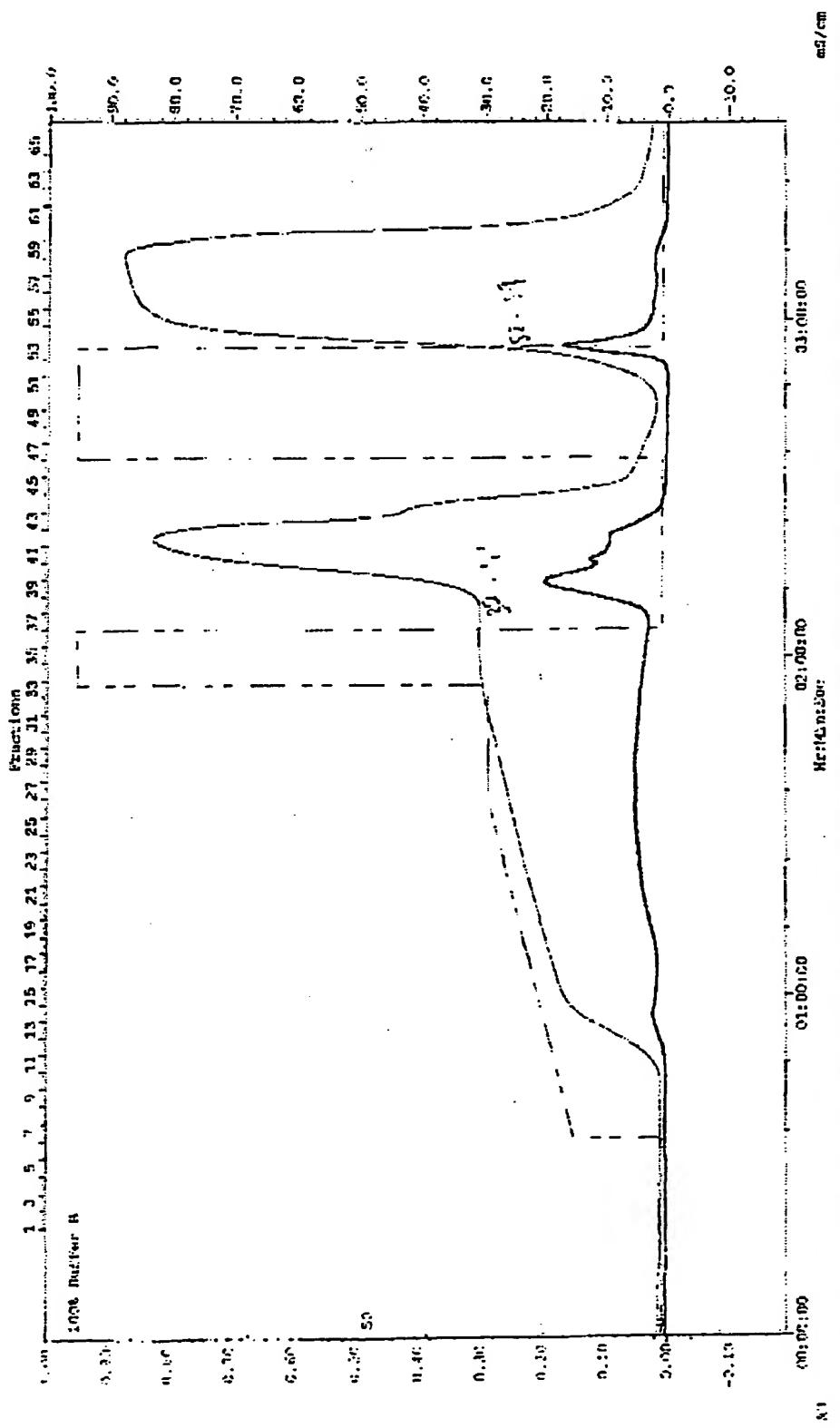
Fraction Size: 3 ml

Run Description: Hum alA + lipid casein falling



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FIGURE 3



Sample: 10 ml. Loaded through pump A

Column: Pharmacia DEAE, 1.6x20 cm.

Buffer A: Tris 10 mM, pH 8.5

Buffer B: Tris 10 mM + 1M NaCl, pH 8.5

Operator: Anki

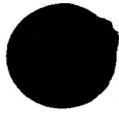
Flow Rate: 1 ml/min

Gradient:

Chart Speed:

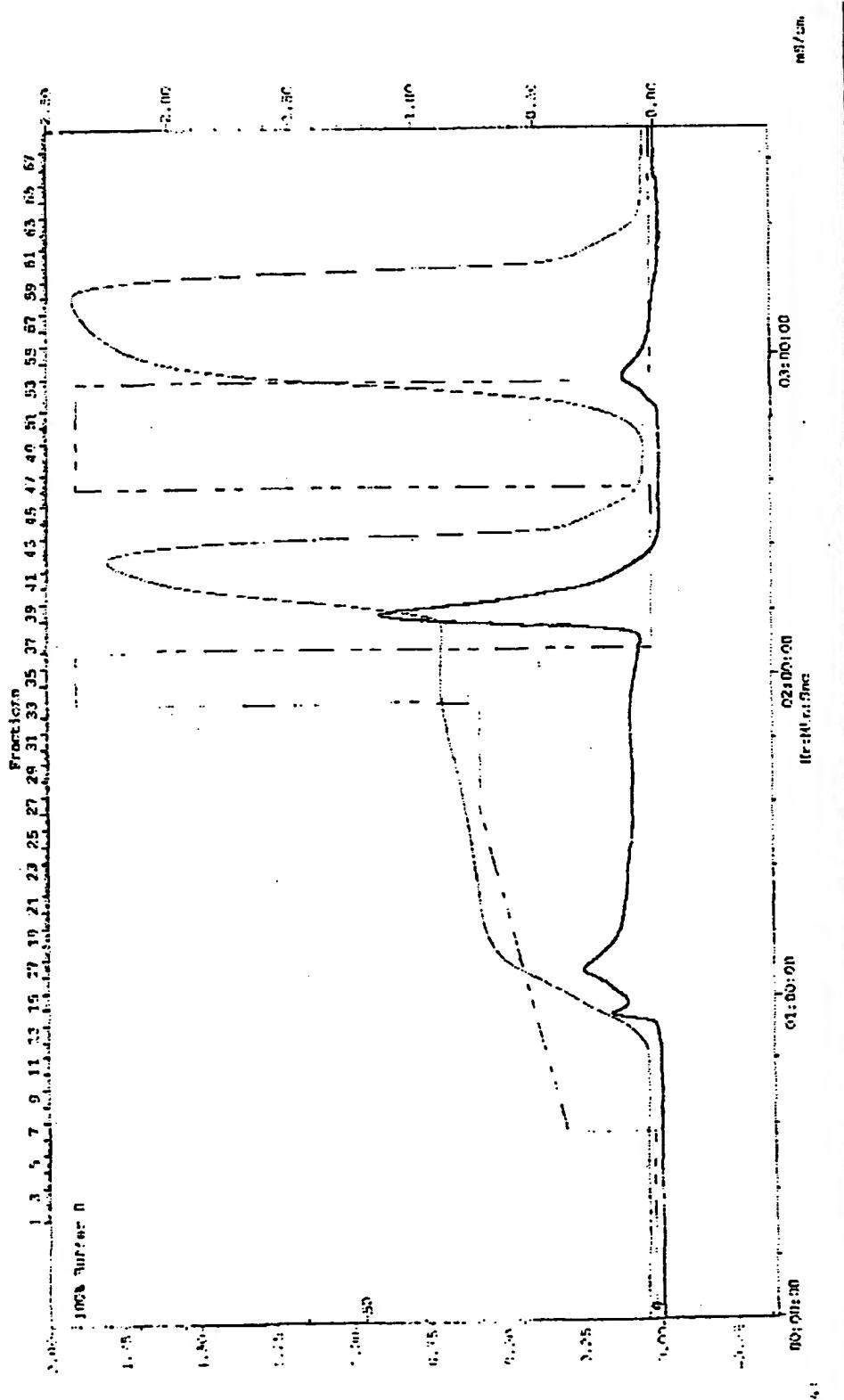
Fraction Size: 3 ml

Run Description: oben Hahntranad pelare



4/4

FIGURE 4



Sample: 10 ml. Loaded through pump A
Column: Pharmacia DEAE, 1.8x20 cm.

Buffer A: Tris 10 mM + 1M NaCl, pH 8.5

Operator: Anki

Flow Rate: 1 ml/min
Gradient:
Chart Speed:
Fraction Size: 3 ml

Run Description: bovin aLA (ca 10mg)

